

Grey matter networks and clinical progression in subjects with pre-dementia Alzheimer's disease.

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Abstract

We studied whether grey matter network parameters are associated with rate of clinical progression in non-demented subjects who have abnormal amyloid markers **in the cerebrospinal fluid (CSF)**, i.e., pre-dementia AD. Non-demented subjects (62 with subjective cognitive decline; 160 with mild cognitive impairment; age = 68 ± 8 years; MMSE = 28 ± 2.4) were selected from the Amsterdam Dementia Cohort when they had abnormal amyloid CSF levels (< 640 pg/ml). Networks were extracted from grey matter structural MRI, and nine parameters were calculated. Cox proportional hazards models **were used to test** associations between each connectivity predictor and the rate of progression to mild cognitive impairment or dementia. After a median time of 2.2 years (1.4-3.1), 122 (55%) subjects showed clinical progression. Lower network parameter values were associated with increased risk for progression, with the strongest **Hazard Ratio** of 0.29 for clustering (95%CI = 0.12 - 0.70; $p < .01$). Results remained **significant** after correcting for tau, hippocampal **volume and MMSE scores**. Our results suggest that at pre-dementia stages, grey matter networks parameters may have use to identify subjects who will show fast clinical progression.

Key words: prognosis, pre-dementia Alzheimer's disease, single-subject, grey matter networks, clinical progression.

1. Introduction

Accumulation of amyloid in the brain is among the first changes leading to Alzheimer's disease (AD) (Sperling et al., 2011; Toledo et al., 2015; van Harten et al., 2013). Future disease modifying therapies are probably most effective in the earliest stages of AD, to prohibit the pathological cascade of events leading to dementia from unfolding. Prognostic biomarkers that can be used to predict time to clinical progression are necessary for disease management, as well as therapy development. Once amyloid is abnormal, it is only weakly related to the rate of clinical progression, probably because it reaches plateau levels at early, preclinical stages of the disease (Jack et al., 2013). Alternative biomarkers are needed that show a better relationship with cognitive decline.

Soluble beta amyloid oligomers and the deposition of amyloid beta into insoluble plaques disrupt synaptic functioning, and loss of synapses has been robustly associated with signs and symptoms of dementia (Selkoe, 2002). Synaptic dysfunction impacts on brain connectivity, and so it can be hypothesised that brain connectivity as measured with neuroimaging techniques might be a sensitive marker for incipient brain damage. One way to measure brain connectivity is based on patterns of coordinated grey matter morphology from structural MRI (Alexander-Bloch et al., 2013a; Bassett et al., 2008; Lerch et al., 2006; Mechelli et al., 2005; Tijms et al., 2012). Coordinated patterns of grey matter morphology have been associated with functional co-activation (Alexander-Bloch et al., 2013b; Andrews et al., 1997), axonal connectivity (Gong et al., 2012), and/or genetic factors (Chen et al., 2013; Schmitt et al., 2009). In AD grey matter networks seem to be more randomly organised, as indicated e.g., by a lower value of the small world coefficient (Friedman et al., 2015; Li et al., 2012; Pereira et al., 2016; Phillips et al., 2015; Tijms et al., 2013a; 2014; Yao et al., 2010; Zhou and Lui, 2013). We previously have shown that worse grey matter network disruptions are associated with worse disease severity and cognitive dysfunction (Tijms et al., 2014; 2013a).

A recent study reported that grey matter network parameters in subjects with mild cognitive impairment (MCI) who later progressed to dementia were more similar to the organisation of grey matter networks in AD subjects, than those of controls (Pereira et al., 2016). In cognitively normal elderly, disruptions of grey matter networks have been associated with more abnormal amyloid-beta 1-42 (A β 1-42) levels in cerebrospinal fluid (CSF), suggesting that grey matter network measures are sensitive to pathological changes at very early stages of AD (Tijms et al., 2016). However, it remains unclear if grey matter network parameters are associated with rate of clinical progression in pre-dementia AD.

In this study, we investigated the hypothesis that grey matter network disruptions are associated with time to clinical progression in a memory clinic sample of subjects with subjective cognitive decline (SCD) or MCI and abnormal amyloid CSF markers. Based on previous studies we expected that indications of a more randomly organised network would be related to a faster clinical decline.

2. Methods

2.1 Subjects

For the COConnectivity in Dementia (CODA) study we selected 62 subjects with SCD and 160 subjects with MCI from the Amsterdam Dementia Cohort (van der Flier et al., 2014) when they had abnormal A β 1-42 CSF levels (<640 pg/ml; see section 2.4 for details of CSF analysis), baseline T1-weighted structural MRI and at least 1 year clinical follow-up data available. Subjects initially visited our memory clinic at the Alzheimer Centre of the VU University Medical centre between 2000 and 2014. Most subjects underwent standard dementia screening that included a medical history, physical and neurological examination, extensive neuropsychological evaluation, screening laboratory tests, an electroencephalogram and an MRI scan. Subjects were diagnosed during a multidisciplinary consensus meeting with SCD when they presented with cognitive complaints, but cognitive and laboratory investigations were normal and they did not meet criteria for MCI, dementia or any other neurological disorder (Jessen et al., 2014); or with MCI when they fulfilled corresponding criteria (Albert et al., 2011; Petersen et al., 1999). For most subjects follow-up visits were part of the regular care, scheduled at about 1 year intervals and included medical history, neurological and neuropsychological work up. Clinical progression was defined as receiving a follow-up diagnosis of MCI or AD-dementia, made in a multidisciplinary meeting based on commonly used criteria (Albert et al., 2011; McKhann et al., 1984; McKhann et al., 2011; Petersen et al., 1999). The primary outcome measure in the present study was time to clinical progression, defined as the time between the baseline MRI and the time that a new diagnosis was made.

2.2 MRI acquisition and preprocessing

High resolution 3D T1-weighted structural images were acquired as part of routine subject care. Over the years, scans were acquired from 7 different MRI scanners (see supplementary material for acquisition details). The distribution of scanner types did not differ between subjects who showed progression and who remained stable ($p > .05$; S-table 1). An experienced neuroradiologist reviewed all scans for brain pathology other than atrophy. Scans were segmented into cerebrospinal fluid, grey and white matter using Statistical Parametric Mapping Software version 12 (SPM12; Functional Imaging Laboratory, University College London, London, UK) run in Matlab 7.12 (MathWorks, Natick, MA). For each subject, 90 brain areas were identified in the native space grey matter segmentations with the use of the Automated Anatomical Labelling Atlas (AAL; (Tzourio-Mazoyer et al., 2002), by warping the AAL atlas from standard space to subject space using inverted parameters that had been calculated for non-linear normalisation of subject images to standard MNI space. Total intracranial volume was calculated as the sum of grey matter, white matter and cerebrospinal fluid voxels from the native space segmentations. Native grey matter segmentations were resliced into 2mm^3 isotropic voxels to ensure equal voxel sizes across all scans.

2.3 Grey matter networks

Single-subject grey matter networks were extracted from native space grey matter density segmentations using a fully automated method previously described in detail (https://github.com/bettytijms/Single_Subject_Grey_Matter_Networks; (Tijms et al., 2012). Briefly, this method determines whether small regions of interest (defined as $3 \times 3 \times 3$ voxel cubes containing grey matter density estimates) show statistical similarity as quantified with Pearson's correlations. A network is constructed by connecting brain areas when the significance of their correlations values exceeds a threshold of $p < .05$ corrected for multiple testing based on permutation testing. Table 1 gives an overview of the network properties that were computed at the node and/or global level for each network: the degree (i.e., the number of edges of a node), characteristic path length (i.e., the minimum number of edges between any pair of nodes), clustering coefficient (i.e., the level of interconnectedness between the neighbours of a node), and betweenness centrality (i.e., the proportion of characteristic paths that run through a node). In addition, the size of the network is the number of nodes in a network, and connectivity density is the proportion of existing edges to the total number of edges possible. To estimate normalised path length λ and normalised clustering coefficient γ , we averaged the characteristic path length and clustering coefficient

across the nodes for each network and then divided these properties by those that were averaged across 20 randomised reference networks that had an identical size and degree distribution (Maslov and Sneppen, 2002). A network is considered to be ‘small world’ when $\gamma/\lambda > 1$. Network properties were computed with scripts from the Brain Connectivity Toolbox that we modified for large sized networks (www.brain-connectivity-toolbox.net, (Rubinov and Sporns, 2010). In order to reduce dimensionality when comparing local network properties, we averaged network property values across nodes within each of the 90 AAL areas.

===== Please insert table 1 about here =====

2.4 Cerebrospinal fluid analysis

CSF samples were obtained with a lumbar puncture between the L3/L4, L4/L5 or L5/S1 intervertebral space using a 25-gauge needle and syringe and collected in polypropylene tubes. Biomarker values were determined at the Neurochemistry laboratory of the department of Clinical Chemistry of the VUmc. A β 1-42 and total tau concentrations were determined with sandwich ELISAs (Innotest, Belgium) (Mulder et al., 2010). Subjects were classified as harbouring abnormal amyloid when CSF A β 1-42 levels were lower than 640 pg/mL (Zwan et al., 2014).

2.5 Statistical analyses

Demographical and clinical characteristics were compared between subjects who remained stable and those who showed clinical progression over time with t-tests, Kruskal-Wallis tests or chi-square tests when appropriate. Baseline network measures were compared for clinical outcome with ANCOVAs, taking into account an interaction effect of baseline cognitive status (SCD or MCI), and as covariates age, gender, normalised whole brain grey matter volume (i.e, grey matter volume divided by total intracranial volume), and scanner type. We used Cox proportional hazards models to assess for each of the 9 global grey matter network measures (predictor variables, i.e, size, degree, connectivity density, clustering coefficient, path length, betweenness centrality, γ , λ and the small world metric) associations with time to clinical progression (dependent variables). All network measures were Z-transformed to aid interpretation of Hazard Ratios (HR). We ran four models: Model 1 included covariates age, gender, normalised whole brain grey matter volume, MRI scanner type and baseline cognitive status (SCD or MCI). Model 2 added CSF tau levels as additional covariate to

Model 1. Model 3 added hippocampal volume (obtained from the AAL parcellation of grey matter) as an additional covariate to Model 2. Model 4 added MMSE scores as an additional covariate to Model 3. If size, average degree or connectivity density showed a significant main effect it was included as an additional covariate, because these properties are known to influence other network properties (van Wijk et al., 2010). Cox proportional hazards analyses were repeated for local network properties in each of the 90 AAL areas, with additional correction for local grey matter volume. Global and local analyses were corrected for multiple comparisons with the false discovery rate (FDR) procedure (Benjamini and Yekutieli, 2001). All statistical analyses were performed in R version 3.2.3.

3. Results

3.1 Sample description

After a median time of 2.2 years, 122 subjects showed clinical progression: 18 to mild cognitive impairment, 96 to AD dementia and nine to other types of dementia (table 2). Compared to subjects who remained stable, those who showed clinical progression more often had MCI, were older, had lower MMSE scores, higher CSF tau levels and **less normalised whole brain grey matter volume**. Stable and progressive subjects had a similar follow up time ($p > .05$). None of the grey matter networks had disconnected nodes and the average connectivity density was 16% (SD = 1.26%).

===== please insert Table 2 about here =====

3.2 Grey matter network measures and clinical outcome in pre-dementia AD

Network size, degree and connectivity density were comparable between subjects who remained stable and those who progressed (table 2). Compared to subjects who remained stable, subjects who progressed had lower γ and small world values. At a local level, subjects who progressed had lower clustering coefficient values in several anatomical areas, but only the left orbitofrontal cortex survived correction for multiple hypothesis testing ($F(1,211) = 15.34$, $p_{FDR} = 0.01$). No interaction effects for baseline cognitive status were found, which suggests that lower γ and small world values associated with clinical progression were similar for subjects with SCD and MCI.

3.3 Grey matter network measures and time to clinical progression

Table 3 shows that the HRs of most network properties were below 1, with lower HRs indicating that per standard deviation decrease in the values of degree, connectivity density, the clustering coefficient, path length, γ , λ and the small world property at baseline, the risk of clinical progression increases. Clustering coefficient values showed the **lowest** HR, with lower values being associated with a 3-fold increased risk for clinical progression (HR = 0.29; 95%CI = 0.12 - 0.70, $p = .006$, $p_{FDR} = .02$). For illustrative purposes, figure 1 shows the clinical outcome for connectivity density, clustering and γ according to tertiles. Results remained largely unchanged after correcting for baseline CSF tau levels, **hippocampal volume** and **MMSE scores** (table 3). Interaction terms of baseline cognitive status and grey matter networks measures on the time to clinical progression were not significant (all $p_{interaction} > .05$), suggesting that the observed associations were similar for SCD and MCI subjects.

Repeating cox proportional hazards analyses for regional network values, additionally correcting for **local grey matter volume**, showed that the association of lower clustering values and time to progression were specific for the right precuneus, left hippocampus and right angular gyrus, occipital areas and the right supramarginal gyrus were associated with time to clinical progression (figure 2). These local associations were subtle however, as none of the local proportional hazards analyses survived correction for multiple testing.

===== Please insert Table 3 about here =====

===== Please insert figure 1 about here =====

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4. Discussion

The main finding of the present study is that in subjects with pre-dementia AD alterations of grey matter network parameters at baseline were associated with faster clinical progression. In these subjects, lower values for degree, clustering, normalised clustering, normalised path length and the small world property, indicative for a more randomly organised network, were associated with clinical outcome and predicted faster clinical progression. Regional analyses suggested that clustering values were lower in specific anatomical areas, comprising mostly temporal and frontal lobes. HRs remained largely unchanged after additional correction for CSF tau, and **hippocampal volume**, suggesting that grey matter network properties have additive value over these more conventional biomarkers. Together, these results provide further support for the hypothesis that disruptions in brain connectivity underlie cognitive decline.

Subjects showing clinical progression over time had lower values for clustering, normalised clustering and the small world property. This suggests that their grey matter networks are organised more like that of a random network, and as such seem to move towards a network organisation that has previously been reported for AD subjects (Li et al., 2012; Pereira et al., 2016; Phillips et al., 2015; Tijms et al., 2014; 2013a) (but also see (Yao et al., 2010) reporting higher clustering in AD and MCI compared to controls). This supports the idea that disruptions in grey matter networks start before the dementia stage of the disease and as such might be sensitive to early changes in brain structural integrity, and are closely related to future cognitive decline.

Associations between lower clustering values and faster decline were found to be specific for several anatomical areas including the inferior parietal gyrus, occipital areas, hippocampus and the precuneus, even when additionally correcting for local grey matter volume. Most of these areas have previously been reported in grey matter network studies to be associated with AD (He et al., 2008; Tijms et al., 2014; 2013a; 2013b; Yao et al., 2010). **The largest difference in local clustering associated with clinical progression was found in the orbitofrontal cortex that is part of the functionally defined 'default mode' network, which seems particularly vulnerable for AD pathology (Buckner et al., 2009). This area is also among brain areas reported to show increased rates of amyloid plaques accumulation subjects with an initially abnormal amyloid positron emission tomography (PET) scan (Villain et al., 2012).** A recent study showed that compared to controls, MCI subjects showing clinical progression had reduced clustering in the right postcentral gyrus (Pereira et al., 2016).

Another study investigating grey matter networks based on correlating rates of change in cortical thickness over time between brain areas, demonstrated improved classification accuracy when differentiating between mild cognitive impairment subjects who remained stable and who progressed over time when the clustering coefficient was included (Li et al., 2012). In particular, clustering coefficient values of the parahippocampal gyrus, temporal lobe and supramarginal gyrus showed the best discriminatory value between groups of stable and progressive subjects (Li et al., 2012). Another study using a similar longitudinal approach (Friedman et al., 2015) showed that network structure in mild cognitive impairment subjects who later progressed to dementia due to AD became increasingly more randomly organised over time. Here we further extend these findings, by showing that grey matter network parameters based on a single baseline measurement are associated with faster clinical progression within amyloid positive subjects.

A potential limitation of the present study is that due to the long period of time during which we were able to retrospectively include subjects, scans were obtained from 7 different scanners that varied in manufacturers and field strengths between 1 and 3T. The use of different scanners might have introduced noise in the data, although it is unlikely that this has biased the present results since stable and progressive subjects showed similar scanner type distributions and scanner type was included as a covariate in the analyses.

In conclusion, in the pre-dementia phases of AD grey matter network alterations can be observed that are suggestive of a change towards a more random network organisation and these alterations seem to predict faster clinical progression. Based on abnormal amyloid, about 50% of pre-dementia AD individuals will develop dementia within a 3-year period. Hence, additional markers are necessary to identify individuals who will progress within e.g., a 1-year period. Here we show that grey matter network parameters might be useful to identify fast progressing subjects, and that in particular the small world property has additive value over more conventionally used biomarkers CSF tau levels and hippocampal volume.

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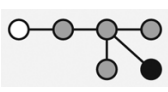
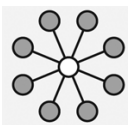
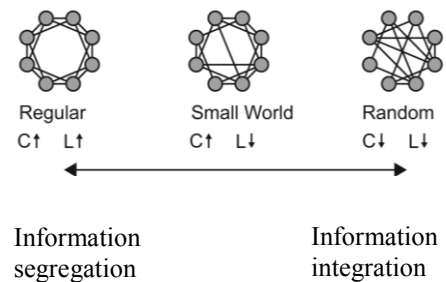
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Figure Legends and Tables

Figure 1. Clinical progression curves for the time to dementia onset in subjects with subjective cognitive decline or mild cognitive impairment for connectivity density, clustering and normalised clustering according to tertiles, adjusted for age, gender, total brain volume, baseline cognitive status and MRI scanner. Clustering and γ were additionally adjusted for connectivity density. Blue lines represent subjects with network property values in the highest tertile, orange with intermediate values and red line with the lowest values.

Figure 2. Surface plots of the AAL areas where reduced clustering was associated with time to progression in pre-dementia Alzheimer's disease subjects. Reduced clustering was associated with time to progression in right supramarginal gyrus, bilateral middle occipital gyrus, right postcentral gyrus, left Heschl's gyrus, left hippocampus, right angular gyrus, right inferior frontal triangularis, right precuneus. All analyses were adjusted for age, gender, total brain volume, local grey matter volume and scanner type.

Table 1. Overview of studied network properties.

Network measure	Global, local	Explanation	Example
1. Network size	Global	Total number of nodes (i.e., brain areas).	
2. Connectivity density	Global	Percentage observed connections from the maximum number possible connections.	
3. Degree	Global, local	The number of connections per node	
4. Clustering coefficient	Global, local	Proportion existing connections between neighbouring nodes from maximum number possible connections. This is a measure of information segregation, i.e., specialised information processing.	The clustering coefficient of the white node would be 0.33, as 1 connection of the 3 possible exists. 
5. Path length	Global, local	Minimum number of connections to go from one node to another node. This is a measure of information integration, as through e.g., long range connections distant clusters can exchange information.	The path length between the white and the black node would be 3. 
6. Betweenness centrality	Global, local	The number of shortest paths that run through a node. This is a centrality measure.	The white node would have the highest betweenness centrality as all short paths run through this node. The network falls apart when this node is removed. 
7. γ normalised clustering coefficient	Global	Quantifies how the global clustering coefficient of an observed network deviates from that of a random network.	
8. λ normalised path length	Global	Quantifies how the global path length of an observed network deviates from that of a random network.	
9. Small world property	Global	An observed network with $\gamma > 1$ and $\lambda \approx 1$ is 'small world', i.e., a network balances specialised information processing through clustering and information integration through long range connections. In a random network path length (L) is minimised, at the expense of a loss of clustering (C). In contrast a completely regularly organised network has high clustering, at the expense of the highest path length value.	

Local properties can be averaged across the nodes of a network to obtain a global description.

Table 2. Baseline clinical and grey matter network characteristics by clinical progression for non-demented memory clinic subjects with abnormal A β 42 CSF markers (cut-off <640 pg/ml).

	Stable	Progression
N (% of total sample)	100 (45%)	122 (55%)
Baseline diagnosis MCI, n (%)	61 (61%)	99 (81%) ^b
Follow up diagnosis MCI, n (%)	n.a.	17 (14 %)
AD dementia	n.a.	98 (80%)
non-AD dementia	n.a.	7 (6%)
Female, n (%)	48 (48%)	61 (50%)
Age years, mean (SD)	67 (8)	68 (8)
Education, median (IQR) [†]	6 (5-6)	5 (4-6)
MMSE, median (IQR)	28 (27-29)	27 (25-28) ^b
T-tau pg/ml, median (IQR)	365 (223-564)	540 (372-803) ^b
Ptau pg/ml, median (IQR)	56 (38-79)	78 (65-108) ^b
Follow-up time years, median (IQR)	2.3 (1.4-3.1)	2.2 (1.3-3.1)
Normalised whole brain grey matter volume, mean (SD) [§]	0.42 (.04)	0.40 (.05) ^a
Network size, mean (SD)	6922 (669)	6822 (706)
Degree, mean (SD)	1129 (126)	1118 (135)
% connections, mean (SD)	16 (1)	16 (1)
Clustering, mean (SD)	0.465 (0.024)	0.463 (0.024)
Path length, mean (SD)	2.02 (0.02)	2.01 (0.02)
Betweenness centrality, mean (SD)	7037.47 (715.53)	6906.57 (715.88)
γ , mean (SD)	1.68 (0.08)	1.65 (0.1) ^a
λ , mean (SD)	1.1 (0.01)	1.1 (0.01)
Small world, mean (SD)	1.53 (0.06)	1.50 (0.07) ^a

% were calculated according to clinical status unless specified otherwise, n.a. is not applicable, MCI is mild cognitive impairment, AD is Alzheimer's disease, MMSE is mini-mental state examination, A β is amyloid beta, SD is standard deviation, IQR is inter quartile range, [§] whole brain grey matter volume was normalized by total intracranial volume, [†] γ is normalised clustering coefficient, λ is normalised path length. [†] data missing for N = 4. Groups were compared using Student's t test, Kruskal tests or chi square tests where appropriate, and for network measures ANCOVAs were

used adjusting for baseline diagnosis, age, gender, normalised whole brain grey matter volume, and scanner type, ^a is $p < .01$, ^b is $p < .001$.

Table 3. Hazard Ratios (95%CI) of Cox proportional hazards analysis of grey matter network property values to predict time to clinical progression in non-demented subjects with abnormal amyloid.

Network property	Model 1	Model 2	Model 3	Model 4
Network size, mean (SD)	1.04 (0.82 - 1.32)	0.58 (0.32 - 1.06)	0.89 (0.46 - 1.72)	0.70 (0.37 - 1.35)
Degree, mean (SD)	0.91 (0.72 - 1.15)	0.94 (0.74 - 1.20)	1.15 (0.88 - 1.50)	1.12 (0.86 - 1.46)
% Connections, mean (SD)	0.74 (0.56 - 0.97) ^a	0.75 (0.56 - 0.99) ^a	0.82 (0.61 - 1.09)	0.76 (0.57 - 1.01)
Clustering [']	0.29 (0.12 - 0.70) ^b	0.30 (0.12 - 0.71) ^b	0.32 (0.13 - 0.78) ^b	0.38 (0.16 - 0.91) ^a
Path length [']	0.75 (0.56 - 0.99) ^a	0.69 (0.52 - 0.92) ^b	0.71 (0.53 - 0.95) ^b	0.75 (0.56 - 1.00) ^a
Betweenness centrality [']	1 (0.78 - 1.27)	1.02 (0.79 - 0.92)	1.24 (0.94 - 1.63)	1.29 (0.98 - 1.70)
γ [']	0.67 (0.52 - 0.88) ^b	0.65 (0.50 - 0.85) ^b	0.70 (0.53 - 0.91) ^b	0.71 (0.54 - 0.93) ^b
λ [']	0.75 (0.57 - 0.99) ^a	0.70 (0.53 - 0.92) ^b	0.72 (0.55 - 0.95) ^b	0.76 (0.57 - 1.00) ^a
Small world [']	0.68 (0.53 - 0.88) ^b	0.67 (0.51 - 0.86) ^b	0.71 (0.55 - 0.93) ^b	0.72 (0.55 - 0.94) ^c

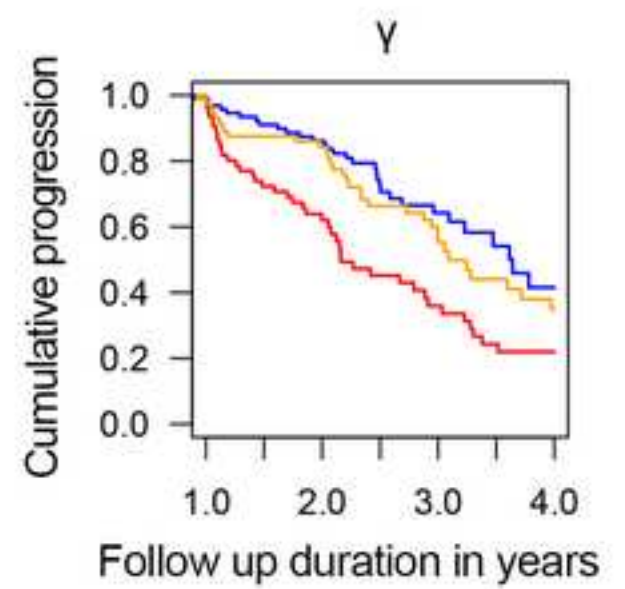
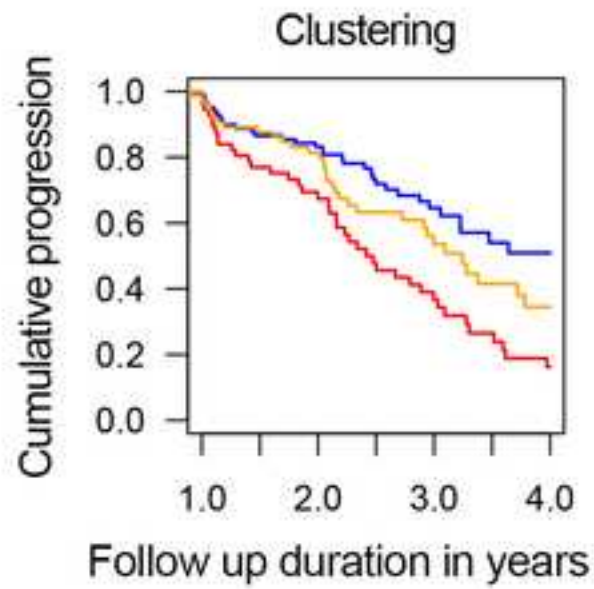
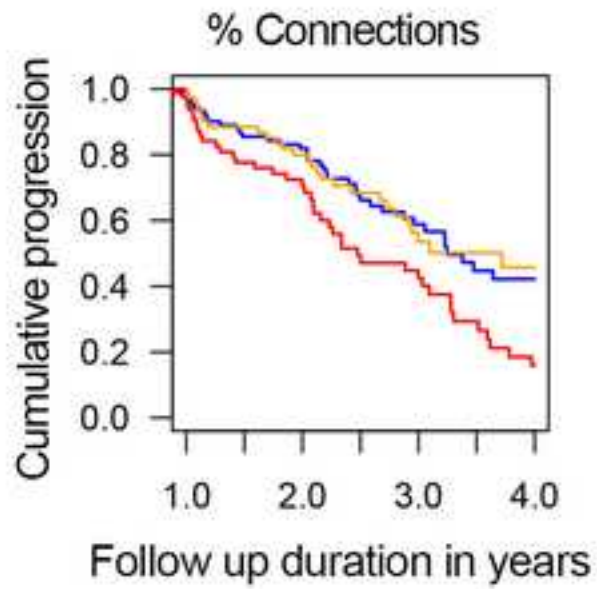
γ is normalised clustering coefficient, λ is normalised path length. All measures were Z transformed and so e.g., Hazard Ratio = .30 means a standard deviation decrease in a network property value is associated with about a 3-fold risk to clinically progress. Model 1: network predictor + age + gender + normalised grey matter volume + baseline diagnosis + scanner type; Model 2: Model 1 + CSF tau levels; Model 3: Model 2 + hippocampal volume; Model 4: Model 3 + MMSE. Variables indicated with '['] were additionally corrected for connectivity density in all models. ^a is $p < .05$, ^b is $p_{FDR} < .05$ with false discovery rate correction for $9 \times 4 = 36$ tests.

Highlights

- Abnormal amyloid is predictive for dementia, but does not predict when.
- Grey matter network alterations were associated with clinical progression
- Low clustering values were associated with 3 fold increased risk to progress
- Grey matter networks may have use to identify subjects who will show fast decline

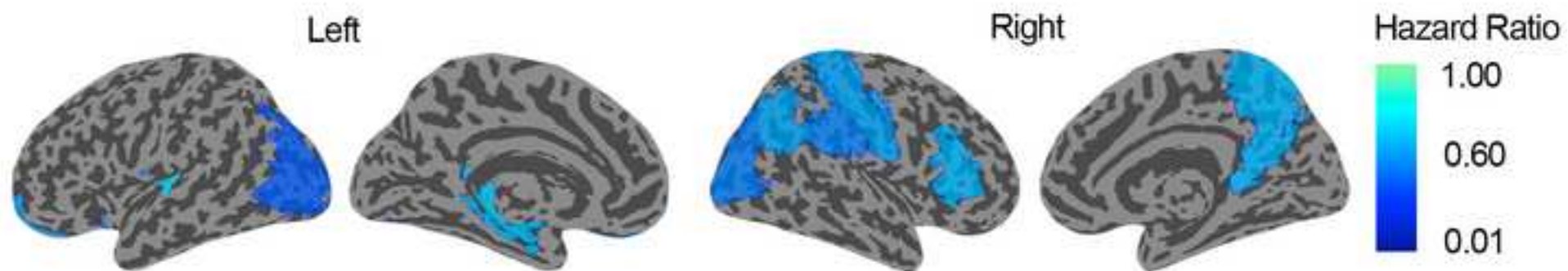
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The data presented in this manuscript have not been previously published and have not been submitted elsewhere, and will not be submitted elsewhere while under consideration at *Neurobiology of Aging*. Appropriate approval and procedures were used concerning human subjects. All authors have reviewed the contents of the manuscript being submitted, approve of its content and validate the accuracy of the data.

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